THE ORIGIN OF EYE SIZE DIFFERENCES IN CICHLID FISH ECOTYPES

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THE ORIGIN OF EYE SIZE DIFFERENCES IN CICHLID FISH ECOTYPES

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LIST OF SYMBOLS AND ABBREVIATIONS

EFTF	Eye field transcription factors
BMP	Bone Morphogenic Protein
WNT	Wingless
LF	*Labeotropheus fuelleborni*
MZ	*Maylandia zebra*
CA	*Cynotilapia afra*
AJ	*Aulonocara jacobfreibergeii*
CB	*Copadichromis borleyi*
MC	*Mchenga conophorus*
M	Mbuna
U	Utaka

SUMMARY

The evolutionary mechanisms that act upon brain and eye development are not well known. Here, we investigate the causes of the adult eye size difference of the two cichlid fish ecotypes from Lake Malawi, and demonstrate that the variation in size starts early in development and is due to early patterning. In brain development, the eye field splits from the forebrain, a process that marks the beginning of the development of the eyes. Genes necessary for eye development include the eye field transcription factors *rx3* and *pax6*. Greater expression of these genes before the eye field first segregates from the forebrain could cause the eyes to be larger when they are initially formed. Of the two cichlid ecotypes, the sand-dwellers (utaka) have larger eyes as adults than the rock-dwellers (mbuna). We show that the utaka have larger eyes than mbuna at the first developmental stage when the eyes are specified, or neurulation. Differences in expression of the eye field transcription factors before neurulation is responsible for this initial size difference.

CHAPTER 1

INTRODUCTION

The forebrain is a complex structure whose organization is generally conserved in all vertebrates. Despite this, it is responsible for many of the attributes we consider uniquely human: conscious thoughts, memories, and emotions. Its complexity adds to the challenging task of understanding how the forebrain forms during embryonic development. Closely coupled with the formation of the forebrain is the development of the eyes. The molecular events that happen in the forebrain that precede the appearance of the eyes show what factors are necessary for their proper formation. In addition, studying the genes involved in eye development can have important implications with regards to human disease.

The formation of the nervous system, or neurulation, begins when the neural plate forms from a thickening of the ectoderm. Changes in cell shape and cell adhesion cause the edges of the plate fold and rise, meeting in the midline to form the neural tube. The forebrain forms from the neural tube, and by the end of somitogenesis it has formed the telencephalon, eyes, hypothalamus, and diencephalon (Wilson & Houart, 2004). Numerous genes and transcription factors are necessary for forebrain development. First, *tlc* expressed from the anterior neural ridge induces the transcription factor *six3*. Six3 is a WNT antagonist that is activated in regions of low WNT activity, and subsequently suppresses Wnt signaling, thereby promoting the formation of the forebrain. Mutations of SIX3 in humans cause holoprosencephaly, a form of cyclopia (Wallis & al., 1999). The specification of the anterior neural plate is also characterized by the action of homeobox-containing genes including *otx2*, *pax6*, and *six3* (Bailey et al., 2004). These, together with *Rx* genes (Retinal homeobox), are eye-field transcription factors (EFTFs)

and are expressed in a dynamic, overlapping pattern of the presumptive eye field of the developing forebrain (Zuber, Gestri, Viczian, Barsacchi, & Harris, 2003). In zebrafish, the homeobox gene *lhx2* is expressed in the presumptive eye field of the forebrain. *Lhx2* and *pax6* cooperate to promote the transcriptional activation of *six6* (Tetreault, Champagne, & Bernier, 2009). In *Xenopus*, *Pax6*, *Six3*, *Rx1*, and *Lhx2* are all first detectable in the presumptive eye field before the completion of gastrulation and the beginning of neurulation (Zuber, et al., 2003).

The eye field is initially unified with the telencephalon in the forebrain, after which the two split from each other. The first morphological evidence of eye formation is the bilateral expansion of tissue from the early forebrain to form the optic vesicles, which starts when the diencephalon moves inward and forward, pushing the eye tissue out laterally (England, Blanchard, Mahadevan, & Adams, 2006; Zuber, et al., 2003). The most distal cells of the optic vesicles form the neural retinal layer (Wilson & Houart, 2004). Loss of *lhx2* function in zebrafish stops eye development before the formation of the optic cup and leads to smaller eyes on the dorso-ventral axis (Seth et al., 2006). At late gastrulation, *rx3* directs cells from the eye field to a retinal fate at the expense of the developing telencephalon, specifically by repressing the telencephalic marker *foxg1* (Stigloher et al., 2006). *Rx3* drives the separation of eye field and telencephalic cells during neurulation, while BMP acts as a repressor of eye-field fate through inhibition of *rx3*, protecting the future telencephalon from acquiring eye identity (Bielen & Houart, 2012). In addition, overexpression of *rx* genes in zebrafish results in the loss of forebrain tissue and the ectopic formation of retinal tissue (Chuang & Raymond, 2001).

Here, I investigate the eye size differences in two ecotypes of cichlid fish from Lake Malawi: the mbuna and the utaka. In Lake Malawi, there are hundreds of species that have recently diverged from a common ancestor and are very ecologically diverse. Their genomes,

however, have stayed highly similar and are comparable to those of any two humans (Sylvester et al., 2010). In adult cichlids, brain size varies and is correlated with ecology and behavior (Sylvester, et al., 2010). For example, algal scrapers (mbuna) have smaller optic lobes, while planktivores (utaka) have relatively larger optic lobes (Huber, van Staaden, Kaufman, & Liem, 1997).

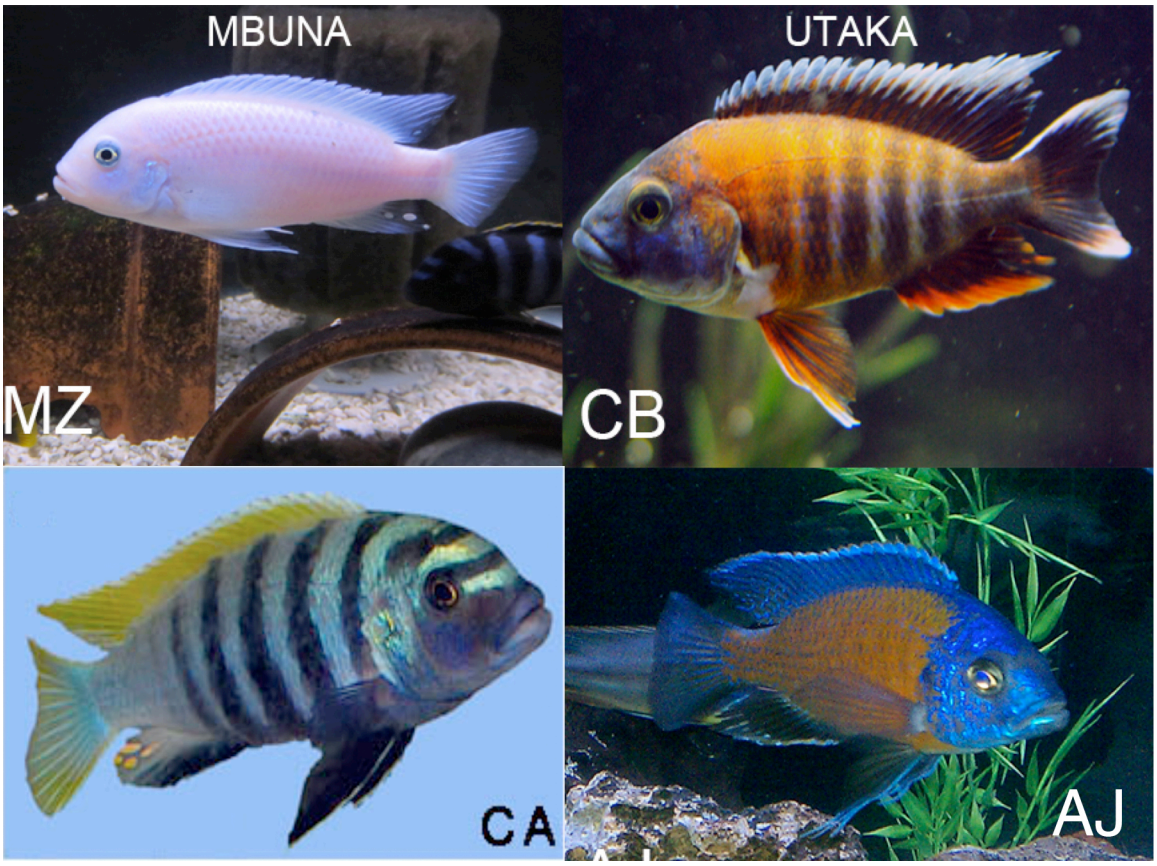

Figure 1. Examples of adult cichlids. Mbuna (left) shown are *Maylandia zebra* and *Cynotilapia afra.* Utaka (right) shown are *Copadichromis borleyi* and *Aulonocara jacobfreibergeii.*

Utaka are also known to have larger eyes than mbuna as adults (Hulsey, Mims, & Streelman, 2007). My project will investigate the developmental origin of these eye size differences. I will do this by first showing a quantifiable difference in eye size in mbuna and utaka during development, and then showing how differences in timing of *rx3* and *pax6* generate larger eyes in utaka. The eyes of the utaka can end up larger one of two ways: (1) the eyes of the

mbuna and utaka are the same size at their first point of specification, but the eyes of the utaka grow throughout development to be larger; or (2) the eyes of the utaka are larger right after specification, due to earlier differences in EFTF patterning, and grow at about the same rate as the mbuna.

CHAPTER 2

MATERIALS AND METHODS

Embryo Raising and Staging.

The embryos of lake Malawi cichlids of multiple species (*C. afra, M. zebra, L. fuelleborni, C. borleyi*, *M. conophorus*, and *A. jacobfreiberg*i) were raised to the required stage at 28°C and were then removed from the mouths of the brooding females. Once at the required stage, the embryos were anesthetized with tricane methanesulfonate and fixed overnight with 4% paraformaldehyde (PFA). Embryos were staged using published protocols (Sylvester, et al., 2010).

Synthesis of RNA Probes for Gene of Interest

Since the cichlid genome was incompletely sequenced at the time, the gene of interest was first found in the zebrafish genome, which is fully sequenced and annexed on Ensemble. BLAST was used to compare the zebrafish gene against partial genome assembly of cichlids to find the closest match to the sequence of the gene of interest in cichlids. Primers were designed for the gene of interest using the website Primer3 (frodo.wi.mit.edu). Primers were designed to produce an amplicon between 500-750 base pairs via PCR. Next, the gene was ligated into a vector and transformed using *E. coli*. This transformation was used to design RNA probes in both SP6 and T7 directions. Both probes were used in a test *in situ* hybridization protocol to determine the sense (same) and anti sense (complementary) directions. Digoxigenin (DIG) molecule was added to the RNA probe mix for labeling purposes.

In Situ Hybridization.

We used digoxigenin (DIG)-labeled antisense RNA probes that were synthesized for the genes of interest using the appropriate cDNA. Whole mount ISH was done by first dehydrating the embryos with methanol and storing them at 20°C, if necessary. Specimens were rehydrated through to PBS with Tween-20 and dechorionated. The probe was allowed to bind at 70°C overnight. The anti-DIG antibody was used at a concentration of 1:3000 and was incubated overnight at 4°C. The color reaction used NBT/BCIP (Roche), with which the embryos were allowed to fully develop the color. Thus, the embryos were continuously transferred into fresh NBT/BCIP solution in NTMT until full staining was reached. After the color reaction, the embryos were washed with PBS and fixed in 4% PFA. Whole mount imaging was taken using a Leica Microsystems stereomicroscope (MZ16). Then, embryos were embedded in chick albumin and gelatin with 2.5% gluteraldehyde. These were post-fixed in PFA and sectioned 20 μm using a Leica Microsystems VT1000 vibratome. Sectioning was done in a frontal plane for eye measuring.

Eye Measurements.

The eyes of mbuna [*L. fuelleborni* (LF), *M. zebra* (MZ), and *C. afra* (CA)] and utaka [*C. borleyi* (CB), *M. conophorus* (MC), and *A. jacobfreiberg*i (AJ)] were measured as a percentage of total forebrain. These species (LF, algal scraper; MZ, generalist; CA, planktivore; CB, planktivore; MC, generalist; AJ, sonar hunter) were chosen to represent the range of the ecological diversity within these evolutionary lineages. Measurements were made from the first developmental stage at which the eyes are beginning to segregate from the forebrain, or neurulation (stage 11), to when the eyes are first distinct from the forebrain, early somitogenesis

(stage 12), to until the eye is completely specified and separate from the forebrain, late somitogenesis (stage 13). Since cichlids are bilaterally symmetrical, only one eye per embryo was measured, assuming that the corresponding eye will have the same eye:forebrain ratio. All measurements were made on scaled digital images of frontal sections using ImageJ software. Means and standard deviations were calculated and a Student's t-test was used to analyze data.

CHAPTER 3

RESULTS

We used cichlid fish embryos from Lake Malawi to ask how the eyes of the two ecotypes, the mbuna and utaka, differ early in development, to cause them to have different sizes as adults. After *in situ* hybridization and sectioning of the eyes of mbuna and utaka, the images were measured for a percentage of eye:forebrain ratio. Utaka were shown to have significantly larger eyes throughout ontogeny (Figure 2). The difference was constant throughout stages 11-13.

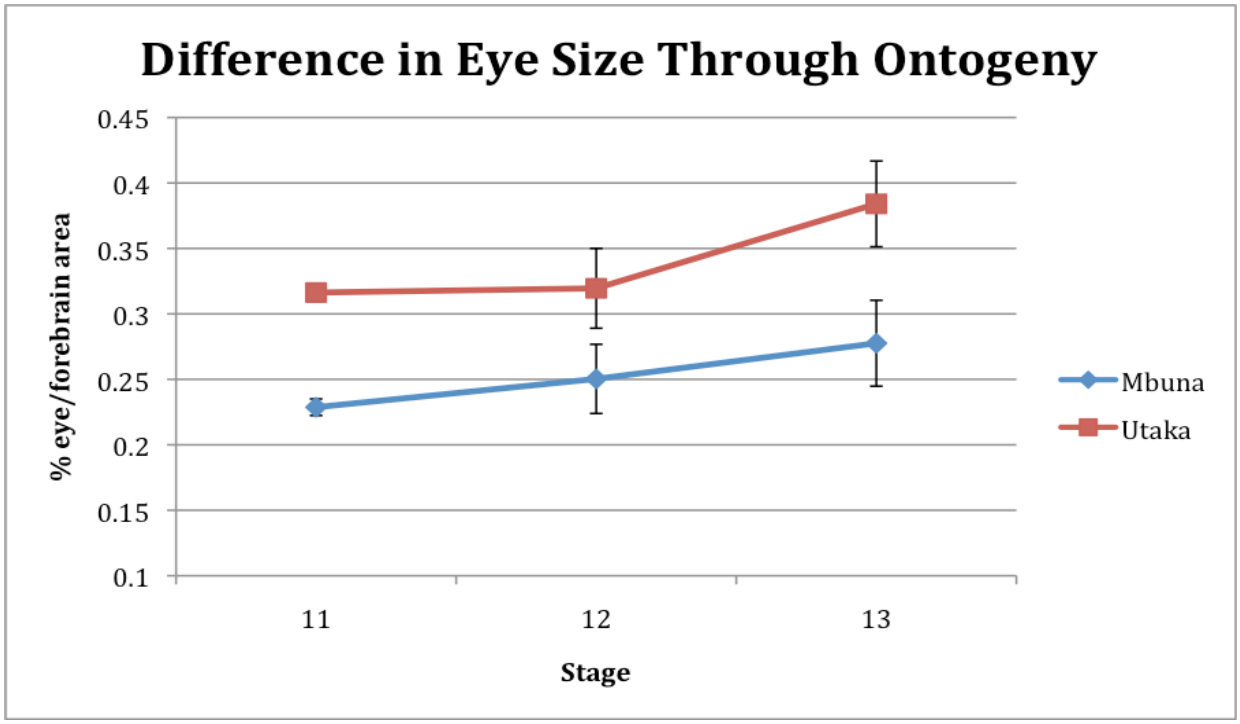

Figure 2. The difference in eye sizes of mbuna (blue) and utaka (red) starts as early as stage 11 and continues until stage 13 (N = 4-9 for each stage). Error bars show standard deviation.

Since the two ecotypes had an initial eye size difference at stage 11, we looked to see how significant this difference was using a Student's T-test. The utaka had significantly larger eyes at stage 11, when the eyes are starting to be specified (Figure 3).

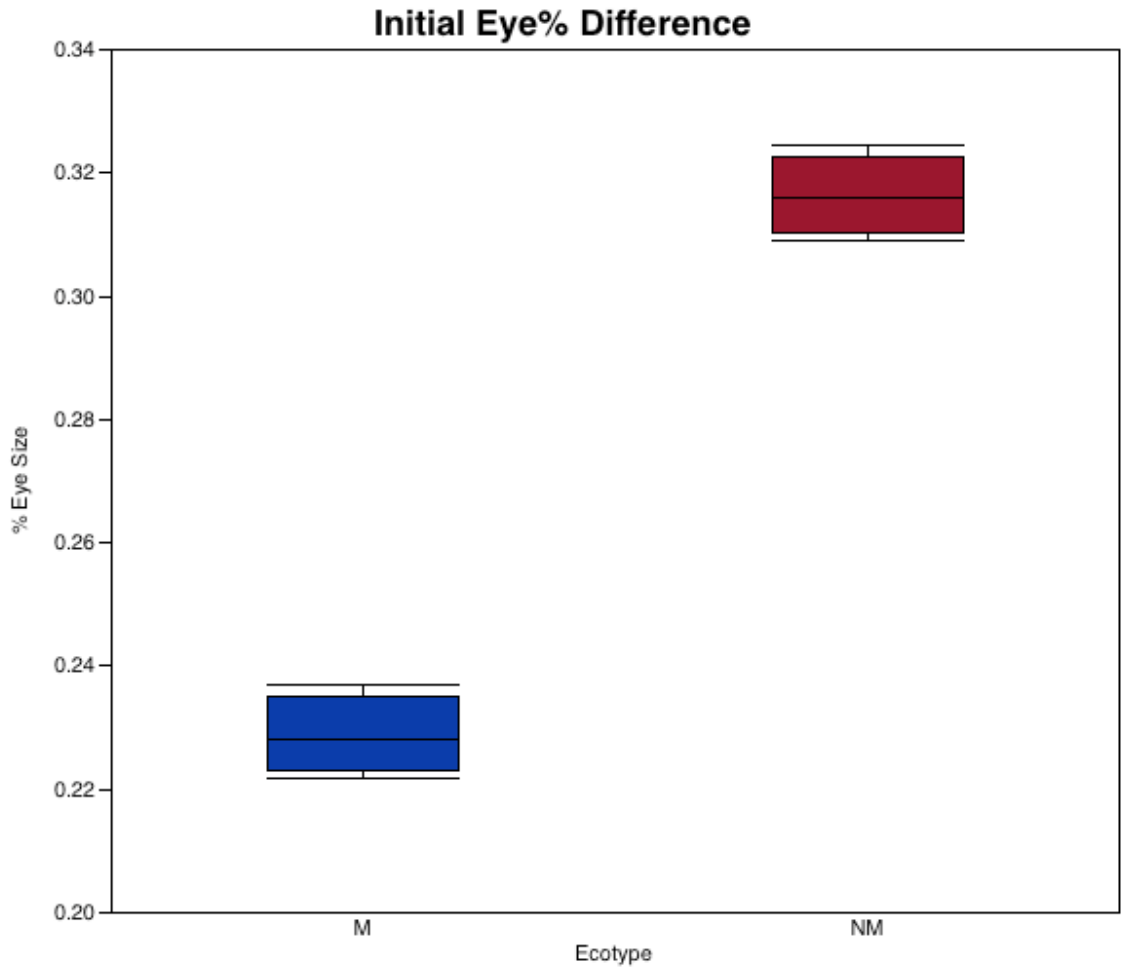

Figure 3. There is a significant difference between the eye sizes of the mbuna (M-blue) and utaka (NM-red) at the earliest developmental stage that the eyes are specified (p = 1.24e-6). Median, minimums, maximums, upper and lower quartiles shown.

The difference in eye size between mbuna and utaka embryos stayed constant throughout ontogeny and was easier visualized at later stages. Figure 3 shows a side-by-side comparison of mbuna and utaka eye sizes in sectioned embryos at stage 13. Many embryos were sectioned and photographed for this project, but it was still challenging to find two embryos, one mbuna and one utaka, that were the exact same stage and size to make this visual comparison. Here, the lenses of the two embryos appear to be similar in size, but the retina of the utaka is visibly larger. The eye of the utaka extends much farther laterally and ventrally than does the eye of the mbuna.

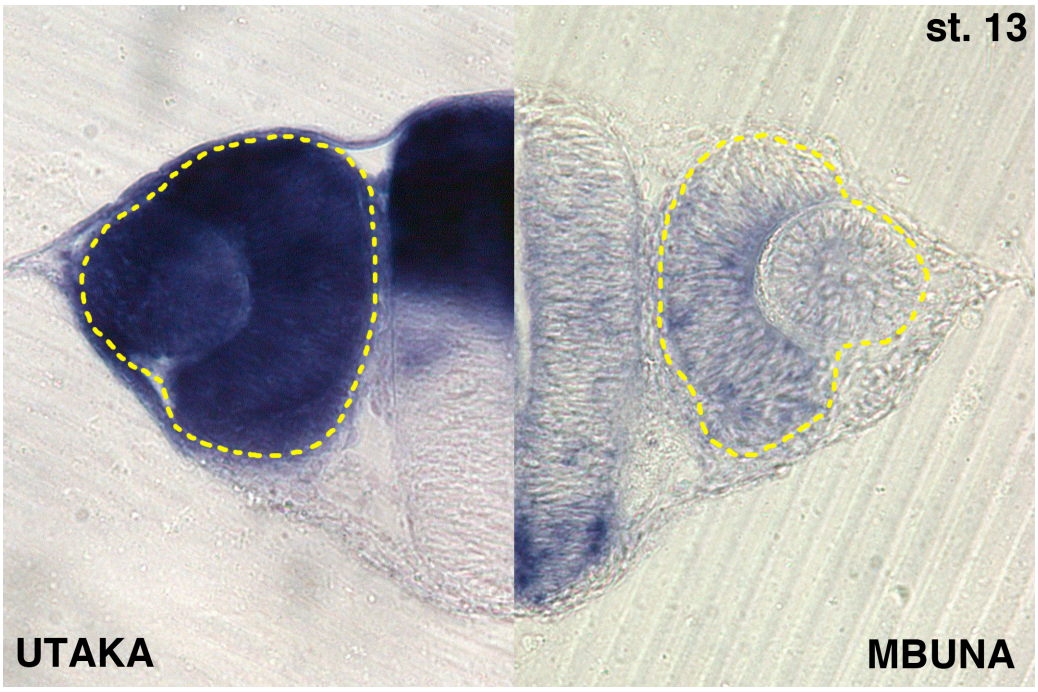

Figure 4. Side-by-side comparison of eye size in sections of utaka (left) and mbuna (right) stage 13 embryos. Yellow dotted line outlines the area of the eye, showing that the utaka's eye is larger. Gene expression shown is *pax6* in utaka and *rx3* in mbuna.

Cichlid brain and eye development was also compared to other vertebrates. Cichlid eye development is similar to other vertebrates, as shown in sections and whole mount photos (Figure 5). Many genes were studied to compare expression in mbuna and utaka and to see their potential roles in eye development. The genes *rx3, pax6, six3, bmp4*, and *lhx2* were chosen for their applicability in brain and eye development in other vertebrates. *In situ* hybridization was performed many times on embryos of varying stages to fully characterize gene expression in cichlids. *Pax6* proved to be useful in visualizing cichlid eye development very well. The segregation of eye field from forebrain tissue is shown in figure 4. The master control eye gene, *pax6*, is important for cichlid eye development from stage 11-14, but most importantly starting with stage 12. *Pax6* is expressed in the developing eyes, as well as the thalamus.

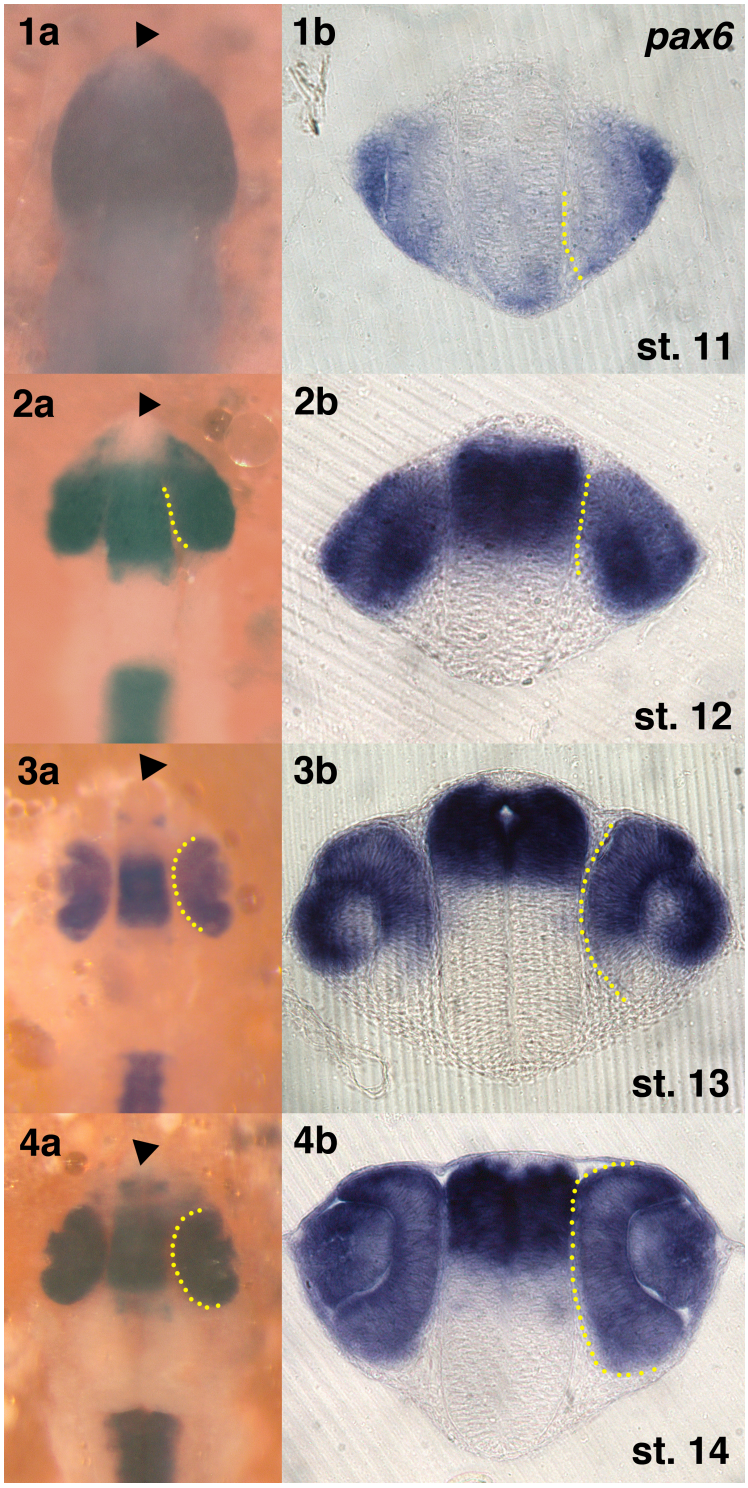

Figure 5. Difference in eye size and expression of *pax6* through ontogeny. Left column (a) shows whole mount (dorsal view, anterior pointing up) and right column (b) shows sections. Arrowheads (panel a) points to forebrain in relation to eyes. **1.** Yellow dotted line (1b) shows the beginning of the separation of forebrain and eyes in section. **2.** Eye field and forebrain are separated further by stage 12, shown by dotted line . **3.**There is a clear separation of eyes from forebrain (yellow dotted lines). **4.** Eyes have clear separation and lenses are more developed.

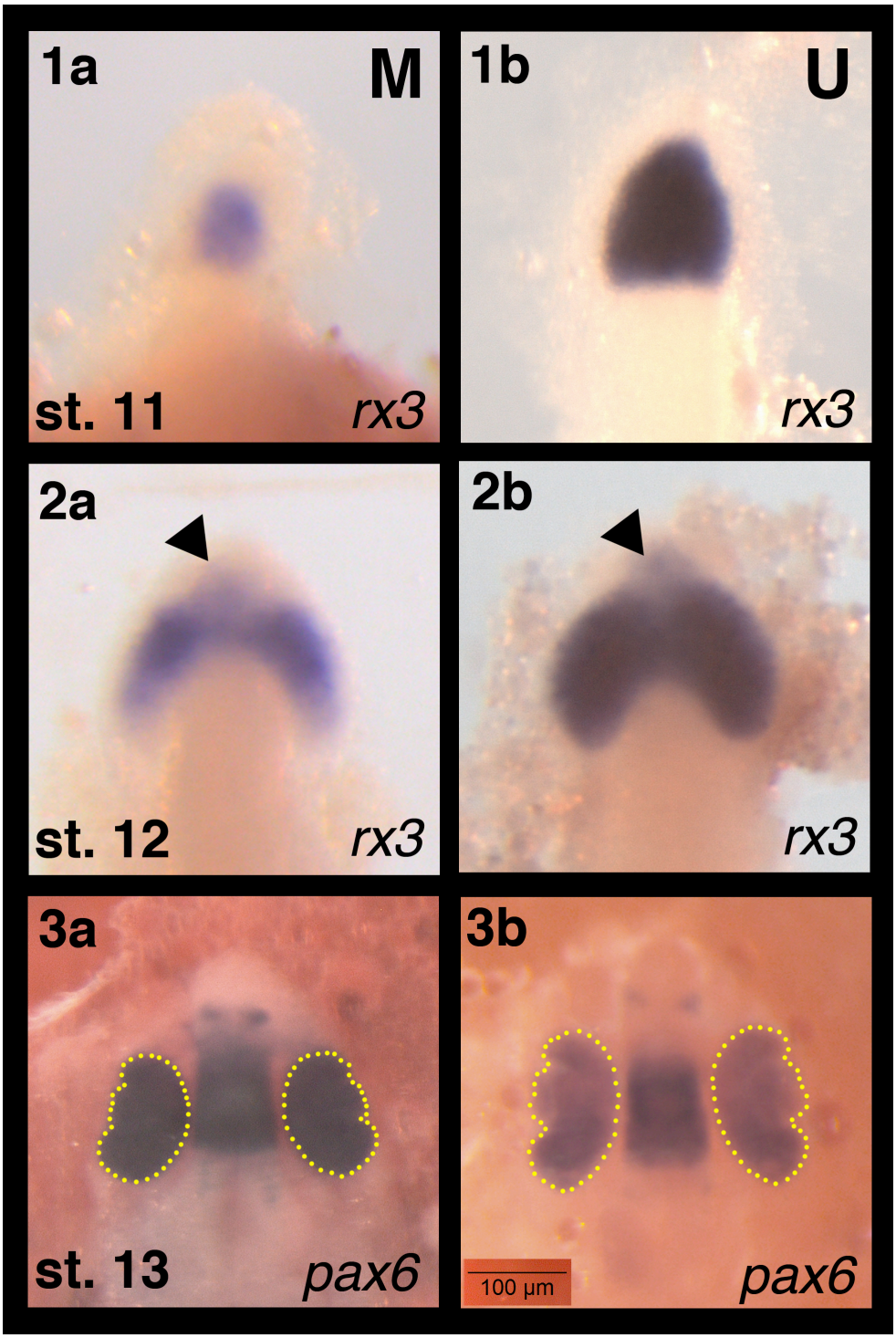

Figure 6. Differences in expression of *rx3* at stages 11-12 and *pax6* at stage 13 in the whole mount dorsal view, anterior pointing up. Panel (a) shows mbuna (M) and panel (b) shows utaka (U). **1.** Greater expression of rx3 in utaka than in mbuna. **2**. Greater expression of *rx3* in utaka than in mbuna at stage 12. Arrowheads show less rx3 expression in the forebrain of the mbuna. **3.** Comparison of eye size in the whole mount, dorsal view. Yellow dotted line shows eye field.

The reason for the initial eye size difference between the mbuna and utaka could be a difference in gene expression as the embryos are developing. The gene *rx3* is important in early eye development, and utaka have greater expression of *rx3* than mbuna at stage 11 and 12 (Figure 6). *Rx3* is expressed wherever developing eye field cells are present, which during stage 11 and parts of 12 is throughout the mixed eye/forebrain area. At stage 12, the eyes are beginning to evaginate outwards, so *rx3* expression follows. *Pax6* becomes important at about stage 13, and is used to illustrate the size difference between mbuna and utaka (Figure 6, 3a-3b). As shown in the dorsal view, the eyes of the utaka extend farther anteriorly, in addition to laterally and ventrally as seen earlier in figure 3.

CHAPTER 4

DISCUSSION

The development of the eyes is closely related to the development of the forebrain. The presumptive eye field starts within the forebrain and later evaginates outward to form the eyes. We used cichlid fish embryos from Lake Malawi to ask how the eyes of the two ecotypes, the mbuna and utaka, differ early in development, to cause them to have different sizes as adults. After *in situ* hybridization and sectioning of the eyes of mbuna and utaka, the images were measured for a percentage of eye:forebrain ratio. Utaka were shown to initially have significantly larger eyes, a difference that stayed constant throughout ontogeny. This confirms our second prediction of the mechanism by which utaka grow to have larger eyes as adults.

The genes *rx3* and *pax6* were found to have the greatest differential expression in the two cichlid ecotypes. Utaka had greater expression of *rx3* earlier in development, at stages 11-12. *Pax6* was helpful later in development at stage 13-14 in visualizing the split of the eyes from the forebrain.

Since the two Lake Malawi cichlid ecotypes have different ecological needs, they understandably have varying anatomy to meet these needs. The rock-dwelling mbuna are algal scrapers and more often use olfaction when feeding. The sand-dwelling utaka live in the open water and are more reliant on sight. It is known that utaka have larger eyes as adults, but this project establishes that the size difference begins as the embryos are developing and as the eyes are first differentiated from the forebrain. Thus, differences in patterning in the early eye field of the embryo could be a factor in determining the adult structures of the cichlid. This can be applied to the development of vertebrates, since cichlid brain and eye development is very

similar to that of other fishes and vertebrates. This improves our understanding of vertebrate brain and eye development by understanding the mechanisms by which evolution can act to increase brain and eye diversity. Knowing the genes involved in eye development and at what stages they are most important can have implications with regards to human disease.

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